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The *Pasteurella* species implicated as the etiologic agent of a massive white perch mortality in the Chesapeake Bay and first described by S. F. Snieszko et al. has been characterized further in our laboratory. The general morphology and physiology of this organism is similar to that of the pasteurellae and several known fish pathogens. There are enough dissimilarities, however, to rule out its identification with any established species. The organism is obligately halophilic and grows in a temperature range between 17 and 31 C on ordinary media containing 1% NaCl. It has a relatively narrow range of *p*H, temperature, and salinity tolerance, and a very short survival time in spent media or brackish water, in contrast to *Pasteurella pestis* and *P. pseudotuberculosis*. Serological tests also indicate that this organism is distinct from other species which it resembles. On the basis of classic morphological and physiological criteria, this organism fits best in the genus *Pasteurella*; the species name *piscicida* (L. noun *piscis*, a fish; L. v.L.adj. suffix *-cidus*, to kill; M.L. noun *piscicida*, fish killer) is proposed.

During the summer of 1963, an epizootic occurred among white perch (*Roccus americanus*) in the upper Chesapeake Bay and its tributaries which destroyed approximately 50% of the population (8). A *Pasteurella* species (designated *Pasteurella* sp. white perch), which appeared to be the etiologic agent, was isolated from the blood and organs of moribund white perch and diseased striped bass (*Roccus saxatilis*) by Snieszko and his co-workers (9). The morphology and physiology of this organism, as well as the rapidly fatal septicemic character of the disease, led to the suspicion that the organism might be closely related to *Pasteurella pestis* and, therefore, potentially dangerous to man. This suspicion was reinforced when a preliminary serological test revealed that rabbit antiplague serum reacted with the organism to form gel precipitin bands.

Because of the possible hazards involved, the work begun by Snieszko et al. (9) was continued in our laboratory at Fort Detrick in order to (i) confirm and extend the identification of *Pasteurella* sp. (white perch), (ii) determine its virulence and pathogenicity in white perch, and (iii) examine its potential danger to public health. The morphology, physiology, and serology of the organism are the subjects of this paper; a study of its virulence, pathogenicity, and epizootiology will be reported in a future communication.

Materials and Methods

Twenty-seven of S. F. Snieszko's original isolates of the *Pasteurella* sp. (white perch) from white perch and striped bass were studied. These isolates were grown on Difco Heart Infusion Broth (HIB) and BBL Blood Agar Base (BAB). Purple Broth (Difco) was used as the basal medium in all sugar fermentation tests. Sodium chloride was added to all media to achieve a final concentration of 1%, except when salt tolerance of the organisms was under study; in this case, Nutrient Broth (Difco) without added NaCl was used as the basal medium. Cultures were incubated statically at 23 C for 48 hr before use.

Cultures of *P. pestis* (virulent Alexander strain) and *P. pseudotuberculosis* (type I strain C-7) from the collection at Fort Detrick were employed where indicated. The cultures of *Haemophilus piscium* and *Aeromonas salmonicida*, as well as the isolates of *Pasteurella* sp. (white perch), were supplied by S. F. Snieszko. The *Vibrio parahaemolyticus* culture was obtained from L. J. Berry, and all other cultures used in the serological studies were from the collection of R. R. Brubaker. All cultures of organisms other than the *Pasteurella* sp. (white perch) were incubated at 26 C on a reciprocating shaker for 48 hr before use.

All tests, techniques, and media used were as recommended in the *Manual of Microbiological Methods* (10). In addition, we also used the following tests: vibriostat 0/129 sensitivity, as described by Shewan et al. (7); coagulase activity with rabbit plasma, as described by Beesley et al. (2); pesticin production

and sensitivity as described by Brubaker et al. (3); and cytochrome oxidase as described by Ewing and Johnson (4).

Phage sensitivity of *Pasteurella* sp. (white perch) was tested by placing drops of phage cultures on agar plates having visible, confluent, surface growth of the bacteria. The phages, which lyse *P. pestis* but not *P. pseudotuberculosis*, were identified in our laboratory as PP1, PP2, PP4, PP5, PP7, PP8, and C-16.

The temperature range in which growth of organisms occurred on the surface of BAB in petri dishes was determined with the method described by Landman et al. (5).

Salinity and *p*H tolerance ranges were determined by preparing duplicate sets of cultures in 100-ml Erlenmeyer flasks, each containing 50 ml of medium adjusted to a certain *p*H or concentration of NaCl. For the salinity test, the medium used was Nutrient Broth (Difco). In the first pair of flasks, no NaCl was added; in the second pair of flasks, 0.5% NaCl was added; and, in the remaining series of flasks, increasing concentrations of NaCl, by 0.5% increments through a maximum of 9%, were added. The same procedure was followed in the *p*H test, except that HIB containing 1% NaCl was used and the *p*H was adjusted by appropriate addition of 0.1 N HCl or NaOH, to 4.5 in the first pair of flasks, to 5.0 in the second pair of flasks, and to *p*H values through *p*H 11 in 0.5 increments in the remaining flasks. After autoclaving the media, the *p*H in one set of flasks was measured and it was assumed that the *p*H was identical in the duplicate set. All flasks were inoculated with 0.1 ml of a 24-hr HIB culture of the indicated organism diluted 1:10,000 with sterile HIB just before inoculation. A series of control flasks were sampled immediately after inoculation, and all of the test flasks were sampled after 48 hr of incubation at 26 C. The number of viable organisms in each sample was determined by the standard pour-plate method with BAB.

Survival of the various *Pasteurella* species in brackish water was determined by inoculating cultures of a representative strain into water from the Chesapeake Bay, at Solomons, Md., which contained 1.7% NaCl. The organisms were grown in 50 ml of HIB; then they were washed free from medium by centrifugation and were suspended in filter-sterilized brackish water twice before finally being dispersed in 200 ml of filter sterilized brackish water. These suspensions were dispensed as 10-ml portions into sterile screw-cap test tubes and were incubated at 23 C. Tubes containing uninoculated sterile brackish water served as a control. An identical test series was also prepared with unsterilized brackish water. Immediately after the test systems were prepared, a single tube from each series of inoculated water and uninoculated controls was sampled and discarded; this procedure was repeated after 1, 2, 3, 4, 5, 14, 21, 28, 42, and 70 days of incubation. The number of organisms surviving after the various time intervals was determined by the standard pour plate method with BAB.

Serological studies involved the use of the gel diffusion analysis method of Ouchterlony (6). Gel plates contained 1% Ionagar #2 (Oxoid) in physiological saline plus 0.1 mg of Merthiolate per ml. Wells were 6 mm in diameter and 9 mm apart (center to center). The plates were incubated at 23 C and were examined after 6, 12, and 24 hr. Bacterial cultures employed as antigens in the serological tests were grown in appropriate media for 48 hr at 26 C, exposed to sonic vibrations for 10 min with a Raytheon sonic oscillator, and sterilized by filtration; then 0.1 mg of Merthiolate per ml was added to the filtrate, which was stored at 5 C until used. Antisera were produced with New Zealand white rabbits by daily intravenous injection (over a 2-week period) of undiluted 48-hr HIB cultures of *Pasteurella* sp. (white perch). The initial injection was 0.2 ml (containing 2×10^8 organisms); then each dose was increased by 0.1 ml until a dose of 1 ml (containing 10^9 organisms) was reached and held constant. Two weeks after the final dose, the rabbits were bled, and 0.1 of Merthiolate per ml was added to the serum before storage at 5 C.

The immunological similarity between *Pasteurella* sp. (white perch), *P. pestis* and *P. pseudotuberculosis* was studied by actively or passively immunizing guinea pigs and mice against *Pasteurella* sp. (white perch) and testing for immunity against lethal infection with *P. pestis*. The animals were injected intramuscularly with 0.1-ml doses containing 5×10^8 organisms. Each of 30 Hartley strain guinea pigs (average weight, 500 g) was injected once weekly for 2 months. Two weeks after the final injection, all of the experimental animals and 10 nonimmunized control animals were each injected intraperitoneally with 235 virulent *P. pestis* cells. Each of 60 Detrick mice (average weight, 20 g) was injected twice weekly for 1 month; 1 week after the final immunizing dose, the animals were separated into six equal groups and were inoculated with graded doses of virulent *P. pestis* by intraperitoneal injection. Also injected with graded doses of virulent *P. pestis* were 60 nonimmunized control mice and 60 mice passively immunized by intraperitoneal injection of 0.5 ml of rabbit antisera against *Pasteurella* sp. (white perch) immediately before challenge. The six challenge doses ranged from 8 to 558 organisms in threefold stepwise increases. In another passive protection test, mice in groups of 10 were each injected intraperitoneally with 0.5 ml of physiological saline, normal rabbit serum, rabbit antiserum against *Pasteurella* sp. (white perch), or rabbit antiserum against *P. pestis*. Immediately after these injections, each mouse was challenged intraperitoneally with 18,000 virulent *P. pestis* cells. All of the actively immunized animals had detectable gel precipitins against *Pasteurella* sp. (white perch); the rabbit antiserum used for passive immunization had a gel precipitin titer of 1:8.

RESULTS

The 27 isolates of *Pasteurella* sp. (white perch) proved to be identical in every respect. When observed in preparations of blood or organs, stained with Geimsa's blood stain, from moribund, experimentally infected white perch (Fig. 1), the bacilli had the typical bipolar safety-pin appearance characteristic of *P. pestis*. The organism grew moderately well in ordinary bac-

FIG. 1. *Pasteurella sp. (white perch) in the blood of a white perch moribund from experimental infection. Giemsa stain. × 1,200.*

teriological media with NaCl added. It was rod shaped with rounded ends, gram negative, encapsulated, nonflagellated, and usually 0.5 by 1.5 μm in size.

Colonies developed within 72 hr on agar media and were uniformly round, 1 to 2 mm in diameter, glistening, greyish yellow, entire, convex, opaque, and viscid. Growth on agar slants was moderately abundant, filiform, glistening, greyish yellow, translucent, and viscid. In Nutrient Broth, the organism produced moderate, uniform turbidity without forming a pellicle and was nonmotile. Colonies on MacConkey's Agar no. 2 (Difco) were uniformly round, 1 to 2 mm in diameter, entire, opaque, convex, viscid, and yellow with a red center. No growth was observed on Difco S-S or Brilliant Green Agar. Stab or slant cultures remained viable for only about 2 weeks when stored at 5 C. Cultures suspended in 60% glycerol plus 40% physiological saline and stored at −20 C survived for approximately 3 months.

Glucose, fructose, maltose, mannose, galactose, melibiose, and sucrose were fermented anaerogenically by the organism, whereas lactose, rhamnose, salicin, sorbitol, xylose, glycerol, mannitol, arabinose, galactose, dulcitol, dextrin, esculin, inositol, melezitose, adonitol, trehalose, inulin, cellobiose, and raffinose were not fermented. Tests for urease, gelatinase, H_2S, indole, acetyl methyl carbinol, and nitrate reduction were negative. The methyl red, cytochrome oxidase, and catalase tests were positive. The organism did not hemolyze rabbit blood, or lyse bovine fibrin, or coagulate rabbit plasma. It did not produce pesticin, nor was it sensitive to this bacteriocin produced by *P. pestis*. Phages which lyse *P. pestis* had no effect on this bacillus. Unlike *P. pestis* and *P. pseudotuberculosis*, it was sensitive to the vibriostat 0/129. A typical strain of *Pasteurella* sp. (white perch) grew in a temperature range of 17 to 31 C, whereas *P. pestis* and *P. pseudotuberculosis* strains were able to grow in a range between 5 and 40 C. At least 0.5% NaCl in media was required for growth of *Pasteurella* sp. (white perch), whereas *P. pestis* and *P. pseudotuberculosis* did not require the addition of NaCl. Optimal growth of *Pasteurella* sp. (white perch) occurred in 1.5% NaCl, with a maximal growth tolerance between 2.5 and 3.0%. *P. pestis* grew best with 0.5% NaCl in the medium, and tolerated 4.5% for growth. *P. pseudotuberculosis* grew best in the medium without NaCl and tolerated 5% NaCl; some organisms survived 48 hr of incubation at 23 C in medium containing 8.5% NaCl. *Pasteurella* sp. (white perch) grew in media having an initial *p*H range between 6.3 and 7.6, with optimal growth at *p*H 6.8. *P. pestis* grew in an initial range between 5.8 and 8.0, with the optimum at 7.2. *P. pseudotuberculosis* grew in an initial range from *p*H 5.2 to 9.3, with an indistinct optimum between 5.8 and 8.9; some organisms survived in medium with an initial *p*H of 10.5.

As indicated in Fig. 2, *Pasteurella* sp. (white perch) died at a logarithmic rate within 3 days when suspended in sterile brackish water (1.7% NaCl). In contrast, *P. pestis* grew during the

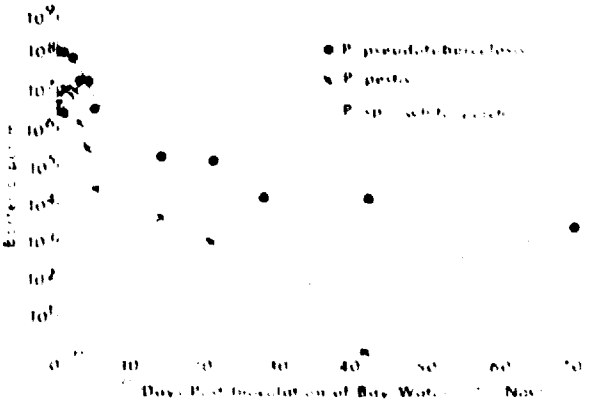

FIG. 2. *Survival of various pasteurella in brackish water.*

first 48 hr and survived between 28 and 40 days under these conditions; *P. pseudotuberculosis* died at an even slower rate, with some organisms still viable after 70 days when the experiment was terminated. Survival of these species in unsterilized brackish water appeared to be similar to their survival in sterile brackish water, although it was impossible to quantitate the number of specific viable organisms accurately because of the large number of indigenous bacteria in the water.

Serum from rabbits immunized with *Pasteurella* sp. (white perch) reacted exclusively with this organism in agar gel double diffusion tests. Two distinct precipitate bands were formed. The serum did not react visibly with the following organisms: *Aerobacter aerogenes*, *Aeromonas hydrophilia*, *A. salmonicida*, *A. shigelloides*, *Escherichia coli B*, *Haemophilus piscium*, *Klebsiella pneumoniae*, *P. pestis*, *P. pseudotuberculosis*, *Proteus morganii*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Salmonella paratyphi A*, *Shigella flexneri*, *Vibrio parahemolyticus*, and *Yersinia enterocolitica*. Rabbit antisera against *P. pestis* or *P. pseudotuberculosis* formed precipitate bands when reacted with *Pasteurella* sp. (white perch), but these bands were not identical to those formed with *P. pestis* and *P. pseudotuberculosis*; furthermore, the sera formed precipitates with most of the organisms listed above. Serum from many nonimmunized rabbits over 6 months of age also formed precipitate bands with most of the organisms listed, including *Pasteurella* sp. (white perch). Serological tests performed by J. Currie at the Walter Reed Army Institute of Research ruled out the possibility that *Pasteurella* sp. (white perch) was a *P. pseudotuberculosis* type, and tests by K. Heddleston at the National Animal Disease Laboratory proved that it was not *P. multocida* or *P. haemolytica* type.

Active or passive immunization of mice and guinea pigs against *Pasteurella* sp. (white perch) had no protective effect against lethal infection with *P. pestis*, in contrast to immunization procedures with *P. pestis* or *P. pseudotuberculosis* as antigens.

The major differences between *Pasteurella* sp. (white perch) and the organisms which it most resembles are summarized in Table 1.

TABLE 1. *Differences between Pasteurella sp. (white perch) and some similar organisms*

Test	*Pasteurella* sp. (white perch)[a]	*P. pestis*	*P. pseudotuberculosis*	*P. [illegible]*	*P. multocida*[b]	*H. piscium*[c]	*A. salmonicida*	*P. haemolytica*
Motility			+					+
Rodent virulence		+	+	+	+			
Gel diffusion test[d]	+							
Growth at 37 C		+	+	+	+			+
Vibriostat 0/129 sensitivity	+							+
Special media[e]						+		
Obligate halophile	+						+	
Soluble pigment	-						+	
NO_3 reduction		+	+		+		+	+
H_2S production		+	+		+			
Urease			+					
Indole					+			+
Fermentation of								
Arabinose				+			+(G)	+
Esculin		+	+				+(G)	
Dextrin		+	+	+		+	+(G)	+
Mannitol		+	+	+			+(G)	+
Rhamnose			+					
Salicin		+	+	+			+(G)	
Sorbitol					+			
Trehalose		+	+		+	+	+(G)	+
Xylose		+	+	+	+			

[a] According to *Bergey's Manual*, 7th ed.
[b] Precipitate forms with serum from rabbits immunized against *Pasteurella* sp. (white perch).
[c] See reference 7.
[d] Requires addition of growth accessory substances to ordinary media.
[e] Indicates fermentation with production of gas.

DISCUSSION

This study confirms and extends the observations of Snieszko et al. (11); it also supports their conclusion that the organism which they isolated from moribund white perch and diseased striped bass should be placed in the genus *Pasteurella*, on the basis of criteria outlined in *Bergey's Manual*. When the organism was compared with the established species which it resembles most closely, there were enough differences in every instance (Table 1) to rule out its identification with any of these species. In addition, no serological evidence that this organism was identical to any of these species was detected. Allen and Pelczar (1) used numerical taxonomy to compare the organism with a large variety of bacteria isolated from the internal organs of white perch, and concluded that it was not significantly similar to any of the organisms tested.

It is our conclusion that the *Pasteurella*-like organism from white perch is not identical to any formally established species. Thus, we suggest that it be identified as *Pasteurella piscicida* (L. noun *piscis*, a fish; L.v.L. adj. suffix-*cidus*, to kill; M.L. noun *piscicida*, fish killer), since it is the only *Pasteurella* species which has been isolated from fish and shown (*unpublished data*) to cause lethal infection of fish.

The *Pasteurella* species from white perch resembles marine bacteria in its absolute requirement for NaCl and in its growth temperature range of 17 to 31 C; yet its range of tolerance to NaCl and hydrogen ion concentration is surprisingly narrow, and its survival time in brackish water or laboratory media is very short, especially when compared to *P. pestis* and *P. pseudotuberculosis*. For these reasons, we suspect that this fish pathogen is poorly adapted for survival outside of its host, and we suggest that this may be a major reason why it was not detected before and has not been detected since the massive white perch mortality occurred in the Chesapeake Bay area in 1963.

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